



The similarity of galactomannan in seeds and endocarp of pods during development in *Senna macranthera* var. *nervosa*

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Abstract

The growth and development of the pods and seeds of *Senna macranthera* (Colladon) var. *nervosa* (Vogel) Irwin & Barneby were morphologically and anatomically studied, and the variation in soluble carbohydrates and galactomannan deposition analyzed. Based on the morpho-anatomical changes, the developmental process was subdivided into 10 stages from anthesis until maturation. This allowed the sampling of fruits of similar development in each phase, in spite of the various stages attained by different fruits at every period of time. Aqueous ethanol-soluble carbohydrates increased gradually in the pods and decreased in the seeds during the process, the opposite occurring with the hot water-soluble ones. A massive deposition of galactomannan in seeds was observed in the phases immediately prior to seed and pod dessication. The mannose:galactose ratio of the seed and pod galactomannans varied from 1:3 in the beginning of deposition to 3.5:1 in the mature dry organs. It is shown for the first time that polysaccharides of the same composition and mannose:galactose proportions of seed galactomannan were detected in the pod (up to nearly 10% of dry mass) prior to its increase in the seeds. Considering the known functions of galactomannans, we suggest that pod galactomannan of *S. macranthera* var. *nervosa* could provide protection and maintenance of adequate humid environment for the development of seeds.

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1. Introduction

Seed development proceeds through histodifferentiation and accumulation of reserves, which end with desiccation that leads to embryo survival in a quiescent stage. In legumes, seeds develop within pods that play a critical role in the transfer of assimilates and other nutrients to the developing embryo. Besides protecting the seeds during development, pod cell walls are part of the source-sink pathway that delivers storage products to the filling seed (Gallardo et al., 2003; Thorne, 1979; Wang and Crusak, 2005).

The endosperm of many legume species was shown to contain galactomannans as cell-wall storage polysaccharides, the amount and composition of which vary from species to species (Buckeridge and Dietrich, 1990; Buckeridge et al., 1995, 2000; Dea and Morrison, 1975). Galactomannans are hemicellulosic polysaccharides composed of a 1,4- β -linked D-mannan backbone to which single D-galactosyl units are attached to C-6 of some of the D-mannosyl residues. The major difference between galactomannans from different seed species is the ratio of D-galactose to D-mannose (Buckeridge, 2010 and refs therein). Besides playing two major biological roles as storage polysaccharides for the growing seed and as structural components of the hemicellulose–cellulose network, galactomannans protect the developing axis from fluctuations in water balance because of its hydrophilic nature (Schröder et al., 2009).

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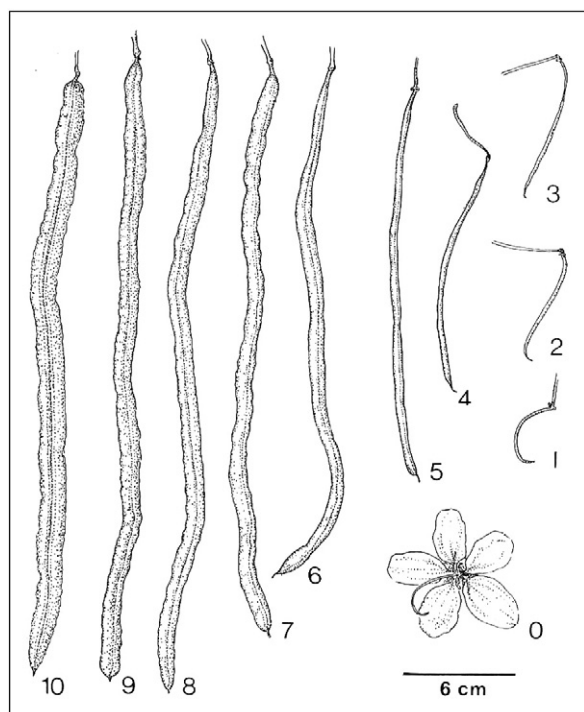


Fig. 1. Aspect of flower and fruits of *Senna macranthera* var. *nervosa* in different stages of development. Numbers refer to characteristics presented in Table 1.

In spite of the great number of galactomannan-containing seeds already reported (Buckeridge et al., 2000), the concomitant study of morpho-anatomic and biochemical processes involved during fruit and seed development are not frequent. Histochemical analyses of legume development have been described for *Cassia absus* and *C. auriculata* (Suri and Deshpande, 1981), *S. macranthera* var. *nervosa* (Áquila, 2004), *Senna corymbosa* (Rodríguez-Pontes, 2007) and *Medicago truncatula* (Wang and Crusak, 2005), most of them galactomannan-storing legumes. In legumes, the steps of carbohydrate deposition was pioneering

described for *Trigonella phoenum-graecum* by Meier and Reid (1977). Since then there are only a few detailed accounts of the deposition process occurring during seed development. These refer to *Trigonella foenum-graecum* (Reid et al., 1987), and *Gleditsia triacanthos* (Mallet et al., 1987), both from temperate regions.

S. macranthera var. *nervosa* is a tree species from tropical environment growing from the state of Bahia down to Rio Grande do Sul in Brazil. During flowering and fruit set, the plants display simultaneously a large number of inflorescences with a large number of flowers and fruits in different stages of development. Quiescent seeds of *S. macranthera* were shown to contain about 25% of dry mass as galactomannan with a mannose: galactose ratio of 3:1 (Buckeridge and Dietrich, 1990), similar to other *Senna* species (e.g. *S. occidentalis*, Buckeridge et al., 2000; Edwards et al., 1992). Thus *S. macranthera* var. *nervosa* was a choice of tropical tree species to undertake physiological and biochemical studies of carbohydrate deposition during pod and seed development, monitored by histochemical analyses.

2. Materials and methods

2.1. Fruit and seed source

Fruits of *S. macranthera* (Colladon) var. *nervosa* (Vogel) Irwin & Barneby were collected from fully grown flowering plants of *S. macranthera* var. *nervosa* cultivated in the campus of the Universidade Federal do Rio Grande do Sul (30°1'59" latitude South and 50°16'11" longitude West), Porto Alegre, RS, Brazil. Flowering and whole fruiting season lasted from March to August (summer and winter, in the southern hemisphere). A voucher of the studied plant is deposited in the ICN herbarium under no. 62566.

2.2. Growth analyses

Flowers were tagged on the day of their anthesis (stage 0) and fruits (pods+seeds) were harvested and separated into 10

Table 1
Main characteristics of fruit and seed developmental stages of *Senna macranthera* var. *nervosa*.
Áquila (2000).

Stages of development	Days after anthesis	Distinctive characteristics
0	1	Ovary before the fall of stamens and petals.
1	2	Ovary after the fall of stamens and petals, dark stigma, zygote and syncial endosperm visible.
2	6	Young fruit about 60 to 70 mm long, zygote without changes and syncial endosperm increasing the number of nuclei.
3	10	Fruit about 80 mm long, slightly curved; embryo starting development, endosperm reaching 36 nuclei
4	14	Fruit about 100 mm long, straight, embryo is globular shaped; endosperm showing beginning of cellularization
5	18	Fruit about 170 mm long, straight; embryo heart shaped, endosperm half cellularized and half haustorial.
6	22	Fruit about 220 mm long, straight, embryo differentiated in cotyledons and axis, endosperm hyalin and fluid.
7	28	Fruit about 230 mm long, slightly striated; embryo fleshy, green, straight; jelly endosperm.
8	38	Fruit about 260 mm long, sinuous, with corrugated surface; green embryo showing the beginning of the axis bending; spongy endosperm.
9	52	Fruit fully formed about 350 mm long, deeply sinuous; fleshy green embryo with bent axis; endosperm fully cellularized and compact.
10	120	Dry fruit with no changes in size and with quiescent, hard brown seeds, vitreous endosperm.

groups according to their developmental stage, determined by their external morphology (Fig. 1) as well as by the seed morphology (Table 1). Seeds were separated from each fruit, measured (length and diameter) using a microscope with a graduated ocular for the initial stages (from 0 to 2) and a stereomicroscope and a rule for the subsequent ones, weighed (fresh mass), and dried in an oven at 60 °C until constant weight (dry mass). Fruit length was measured using a flexible wire, to follow the fruit shape, and subsequently distended and measured with a rule. Fruit width was measured with a pachimeter. Pods (fruits without seeds) were also weighed and dried. Fifty fruits and fifty seeds were measured for each group.

2.3. Histochemical analysis

Fragments collected from pods and seeds were fixed in FAA for 48 h (Johansen, 1940). Subsequent dehydration in isopropyl alcohol series and paraffin embedding were performed according to Engleman (1979). Microscopic observations were made in 6 to 12 µm-thick slices of seeds and pods cut using a rotative microtome, mounted on glass microscope slides, and stained with Clorazol black E (Curtis-Patiño, 1986) and/or PAS (McManus, 1946), in a Jenamed Microscope Carl Zeiss, as previously described (Aquila, 2004).

2.4. Carbohydrate extraction and analysis

Carbohydrate extractions were performed in triplicate from aliquots of combined samples of 50 seeds or 10 pods. Seeds were extracted only from stages 6 up to 10 since in earlier stages they were too small and insufficient to compose the samples. Therefore, from stage 1 to 5 only pods were extracted and analyzed. Oven dried seeds and pods were ground in a micromil (MR Manesco & Ranieri Ltda.) and 2 g of powder were then defatted with benzene:ethanol 2:1 (v/v) under reflux for 24 h. Soluble sugars were exhaustively extracted from the dried defatted powder in 80% ethanol (20 mg ml⁻¹) at 80 °C for 15 min and separated from the insoluble residue by centrifugation at 3000 g for 15 min.

The galactomannan-containing residues were subsequently suspended in water (1 g l⁻¹), and extracted at 80 °C for 12 h, filtrated, precipitated with two volumes of ethanol according to Anderson (1949), dried, and weighed. The crude galactomannan was powdered, hydrolyzed in 4 N TFA (Buckeridge and Dietrich, 1990), acetylated (Albersheim et al., 1967) and submitted to GC as described by Buckeridge and Dietrich (1990). Galactose, mannose, arabinose, xylose, and glucose from Sigma Chem. Co. (USA) used as standards were submitted to the same acetylation procedures as the galactomannan prior to GC.

Aqueous ethanol-soluble oligosaccharides were identified by descending paper chromatography (Jarvis and Duncan, 1974) stained with a silver nitrate reagent (Trevelyan et al., 1950), using sucrose, melibiose, and raffinose as standards. Total and reducing sugars were quantified by the phenol-sulfuric (Dubois et al., 1956) and Somogyi Nelson's procedures (Somogyi, 1945) respectively, using glucose as standard.

2.5. Statistical analysis

Data were subjected to variance analyses by ANOVA followed by the Duncan test at 5%.

3. Results

3.1. Fruit and seed growth

The early stages of development of fruits and seeds of *S. macranthera* var. *nervosa* are characterized by slow growth (width) and slow increase of dry and fresh mass (Fig. 2). The fruit starts the exponential phase of growth about 14 days after anthesis and the seed immediately after that. The net gain in weight (Fig. 2E–H) correlates to seed and pod elongation (growth) (Fig. 2A–D). After this period and up to about the 55th day (stage 9), the seed growth rate slows down and the endospermic cells become thickened due to the deposition of storage material (Fig. 3A–D). These cells maintain cytoplasmic material which is interconnected via plasmodesms (Fig. 3C). The final stage, which represents the seed maturation period, lasts up to about the 120th day after anthesis. During this period the seeds decrease in size and weight (Fig. 2D–F) and become visibly brownish, hard and impermeable to water, with the layers of reserve polysaccharide deposition distinguishable (Fig. 3D). Histochemical analysis of the pods indicates that during development, similar to the endospermic cells, endocarpic cells become thick-walled (Fig. 3E) and reactive to polysaccharide staining (not shown).

3.2. Carbohydrate content and composition

The yield of aqueous ethanol-soluble substances increased gradually in the pods whereas the opposite occurred with the hot water-soluble ones (Table 2). In seeds, aqueous ethanol-soluble components are low during the whole process and tend to decrease at the final stages of development. On the other hand, hot-water solubles increased up to around 30% of the total dry mass (Table 2).

The carbohydrate content in the ethanolic- as well as in the water-soluble extracts increased during development both in pods and seeds reaching the maximum at the end of the process (120th day after anthesis) (Fig. 4). In the ethanolic fractions, total sugars varied from 15 (stage 0) to 26 mg g⁻¹ of dry mass (stage 10) in pods and 5 to 9 mg g⁻¹ of dry mass (stage 6) in seeds. Nevertheless, the proportions of total and reducing sugars varied differently in these two organs, the total sugar being always higher than the reducing sugars in pods and reaching the total value of sugars in seeds by the end of development (Fig. 4).

The composition of the hot water-soluble polysaccharides varied during fruit and seed development (Fig. 5). In the earlier stages of development (1 to 5), only pods were extracted since seeds were too small and their yields insufficient to compose the samples. From stages 1 to 5 the yield of pod polysaccharides constituted 5.5 to 8.8% of the total dry mass (Table 3). The polysaccharides were composed of galactose,

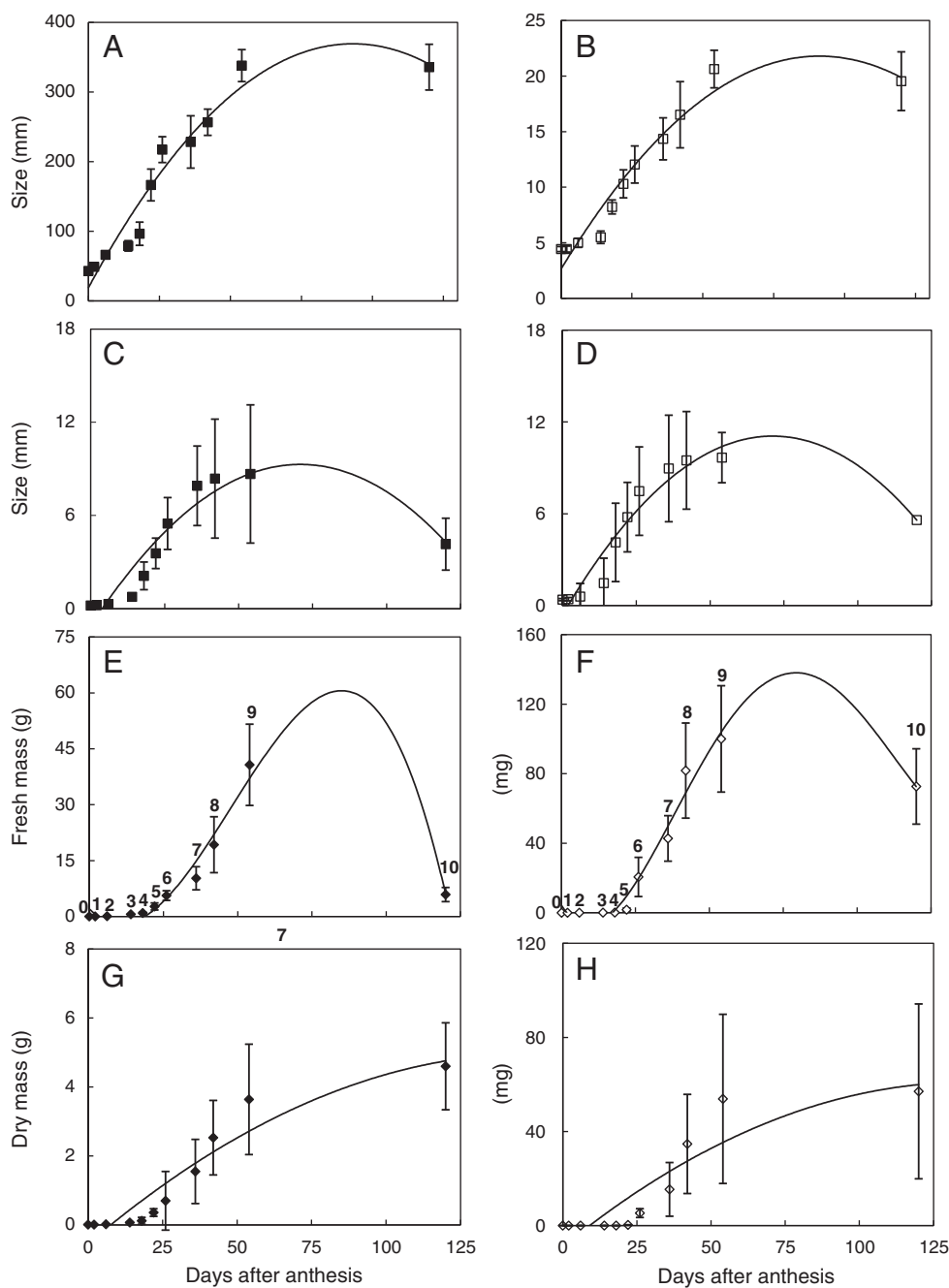


Fig. 2. Growth of (A, C, E, and G) fruits and (B, D, F, and H) seeds of *Senna macranthera* var. *nervosa* during development. (A and B) Length, (C and D) width, (E and F) fresh mass, and (G and H) dry mass. Bars represent the standard deviation of the mean. $n=50$. Numbers inside the figures in E and F refers to the developmental stages presented in Fig. 1 and Table 1.

mannose, ramnose, arabinose, xylose, and glucose, the mannose:galactose ratio being 0.25, indicating the prevalence of galactose (Fig. 5A). During stages 6 to 10, the yield of the polysaccharides decreased in pods and increased in seeds proportionally to the increase in seed weight, attaining 90% of the total extract, i.e. nearly 30% of dry weight at the end of maturation. Seed polysaccharide composition was similar to that found in the pods (Fig. 5B). At the last period, the massive increase in hot-water solubles coincided with galactomannan deposition

(Figs. 3, 4, and 5). The mannose:galactose ratio increased in pod and seed polysaccharides, with high percentage of galactose in the beginning up to 3.5:1 (Fig. 5).

4. Discussion

The various stages attained during the process of fruit and seed formation in *S. macranthera* var. *nervosa* do not bear a simple relationship with the time elapsed after anthesis. This

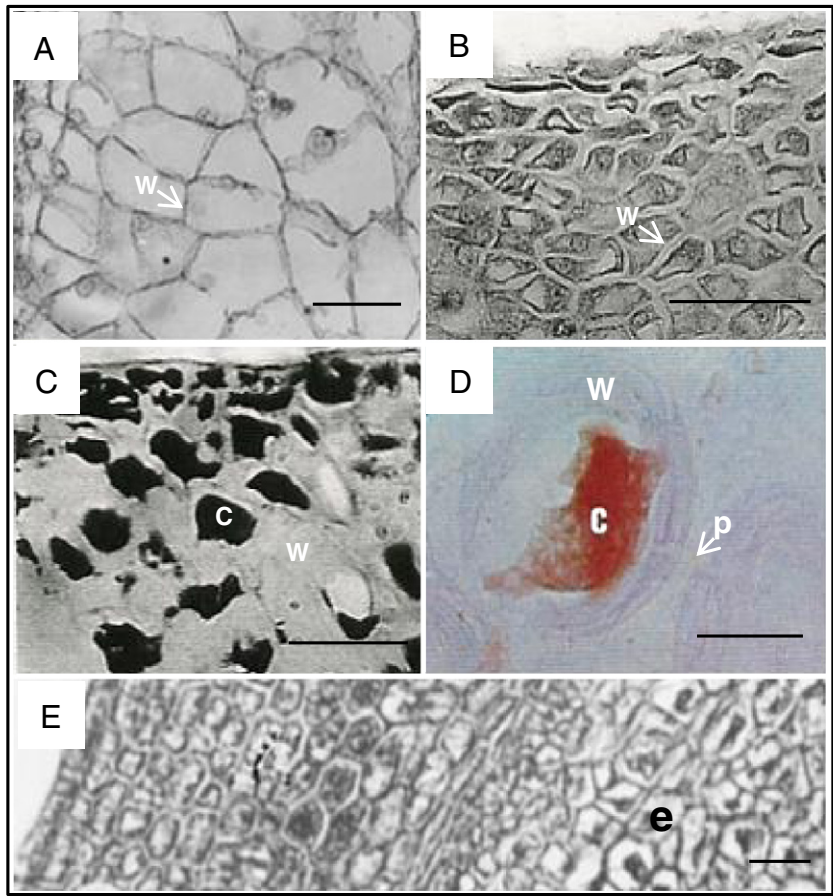


Fig. 3. Anatomical details of (A–D) endosperm and (E) pod of *Senna macranthera* var. *nervosa* during development. (A) Cellularized endosperm (stage 5), (B) thickening cell walls (w) (stage 7), (C) fully thickened cells (stage 10), (D) detail of fully thickened cell of the quiescent seed showing layered walls (w), cytoplasm (c) and plasmodesms (p) after PAS staining; (E) fruit layers showing thickened endocarpic cell walls (e). Bars are A, B, and C=12 μ m, D=8 μ m and E=20 μ m.

Table 2
Yield of aqueous ethanol-soluble substances and polysaccharides from pods and seeds of *Senna macranthera* var. *nervosa* in different phases of development extracted in 80% ethanol and hot water, respectively.

Stage of development	Days after anthesis	% Dry mass			
		Aqueous ethanol-soluble substances		Polysaccharides	
		Pod ^a	Seed	Pod ^a	Seed
0	1	7.27 a	–	5.37 a	–
1	2	7.34 a	–	6.61 a	–
2	6	8.31 b	–	6.73 a	–
3	10	8.61 b	–	7.31 ab	–
4	14	9.62 b	–	7.67 ab	–
5	18	9.83 b	–	8.61 bc	–
6	22	12.33 c	31.24 a	9.34 cd	5.17 a
7	28	14.29 d	20.86 b	9.75 cd	13.52 b
8	38	20.66 e	17.22 c	8.37 cd	18.43 c
9	52	23.29 e	16.35 c	6.79 a	28.60 d
10	120	26.89 f	15.42 c	6.50 a	30.10 e

Letters compare means of triplicates (composed samples of fifty seeds or 10 pods) in each column by Duncan ($P<0.05$); – not determined.

^a From stages 0 to 5 only pods were analyzed due to the low yield of seed contents.

requires a more consistent referential, such as the morphology and physiology of these structures. In the genus *Cassia*, part of which is now classified as *Senna* or *Chamaechrista* (Irwin and Barneby, 1982), the embryology was described for several species (Oliveira, 1999; Rodriguez-Pontes, 2007; Suri and Deshpande, 1981; among others). The content and composition of reserves in mature seeds have also been described and analyzed, particularly for legumes (reviewed by Buckeridge et al., 2000). However, very few reports on changes occurring during their development have appeared (e.g. Meier and Reid, 1977 for *T. foenum-graecum*, Mallet et al., 1987 for *G. triacanthos*, Marangoni and Alli, 1988 for *Prosopis juliflora*, Wang and Crusak, 2005 for *M. truncatula*). Most of these reports are concerned with the seed parts. Stems and pod-stored assimilates were considered not available for the developing seed (Evans and Wardraw, 1976; Hume and Criswell, 1973) until Thorne (1979) demonstrated the role of pods as transient reserves for seed maturation.

In *S. macranthera* var. *nervosa*, the growth and dry matter content in pods and seeds follow the same patterns from anthesis up to maturation (Fig. 2). This suggests a continuous flow of nutrients from the pod to the seeds as suggested by

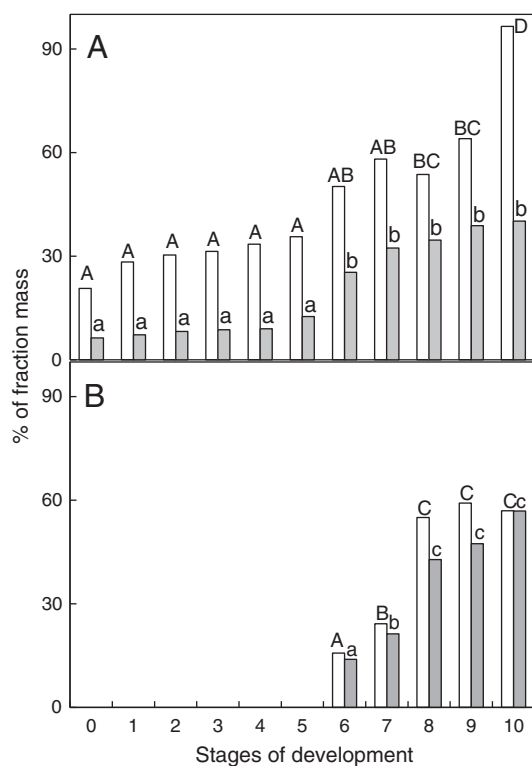


Fig. 4. Variation of total and reducing sugars in (A) pods and (B) seeds of *Senna macranthera* var. *nervosa* during development. Numbers refer to stages presented in Table 1. Letters compare means of triplicates (composed samples of fifty seeds or 10 pods) by Duncan 5% after transformation of the % values in arc sin.

Thorne (1979). Indeed, the aqueous ethanol-soluble carbohydrates sucrose, glucose, fructose and melibiose (detected by paper chromatography, not shown) are found in pods and seeds as well. However, as judged by the difference between total and reducing sugars, sucrose seems to be the major component of the ethanol carbohydrates in pods, whereas in seeds both components are present in nearly equivalent and low proportions (Fig. 4). In both pods and seeds, the level of sugars showed a slow increase up to stage 7, when there is a significant increase in pods and a decrease in seeds. This probably coincides with the closure of the connection between seeds and pods as suggested by Áquila (2004). This means that pods are possibly supplying the substrates for seeds that utilize them for the synthesis of their reserves.

In the present work we have found that galactomannans stored in the endosperm of mature seeds of *S. macranthera* var. *nervosa* are also deposited in the pods of this species (Fig. 3), bearing the same mannose:galactose proportions as those found in the seeds (Fig. 5). In the pods, these hot water-soluble polysaccharides, presented here for the first time, seem not to function as reserves, since they are present in relatively low amounts and remain at approximately the same amounts in this organ until the end of fruit development (Table 3). At the early stages of fruit development, both pod and seed stored polysaccharides contain a relatively high proportion of galactose that gradually decreases to attain a proportion of 3 mannose:1 galactose, which is also the same proportion attained in the seeds

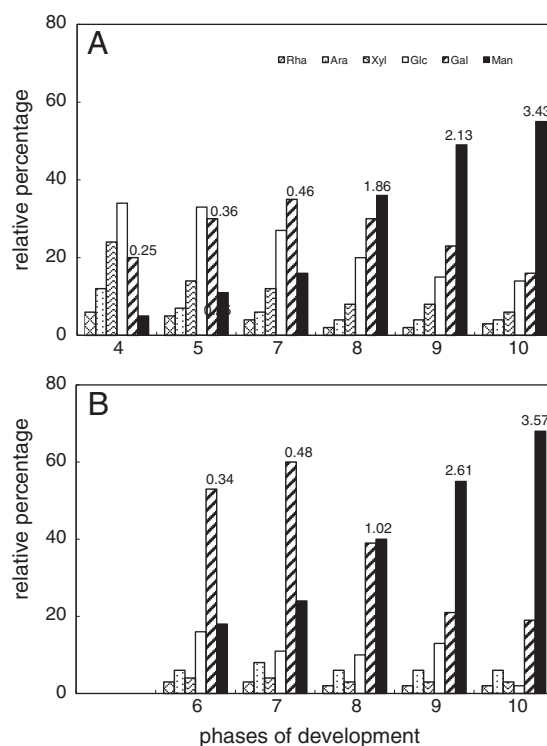


Fig. 5. Proportions of sugar components from acid-hydrolyzed polysaccharide fraction from (A) pods and (B) seeds at different stages of development. Numbers on top of bars represent the mannose:galactose ratio in each stage. Numbers refer to stages presented in Table 1. Data represent means of triplicates (composed samples of fifty seeds or 10 pods).

at the end of maturation. This suggests that both organs contain the same metabolic components to synthesize the polymer. Edwards et al. (1992) have shown in *S. occidentalis* that the ratio of activity of mannosyltransferase/galactosyltransferase increased substantially during the period of galactomannan deposition. Activity of α -galactosidase also increased in the endosperm in late galactomannan deposition, suggesting a cause–effect relationship between the increase in the mannose:galactose ratio and the developmental pattern of the enzymes. Therefore changes in the mannose:galactose ratio in seeds of *S. macranthera* var. *nervosa* could be due to a similar enzymatic control of the galactomannan biosynthetic process. Therefore, it would be interesting to clarify whether the same process leading to galactomannan biosynthesis is taking place in the seed endosperm and pod, also including the more recently described galactomannan transglycosidase (Buckeridge, 2010).

Since Reid and Bewley (1979), it has been suggested that the galactomannan is a multifunctional molecule in seeds, providing cell wall strength, accelerating the imbibition process and preventing embryo desiccation during germination besides providing energy and substrate for embryo growth. Maybe some of these functions are also exerted by pod galactomannan in *S. macranthera* var. *nervosa*, providing protection and maintaining an adequately humid environment around the developing seeds, thus preventing their premature water loss and consequent abortion.

Table 3
Yield of hot water-soluble constituents of pod and seeds of *Senna macranthera* var. *nervosa* in different stages of development.

Stage of development	Days after anthesis	Pod			Seed		
		Yield		Man:gal ratio	Yield		Man:gal ratio
		(g±s.d.)	%		(g±s.d.)	%	
0	0	0.11±0.01 a	5.5	0.25	–	–	–
4	14	0.15±0.02 ab	7.5	0.25	–	–	–
6	22	0.19±0.01 cd	9.5	0.36	0.10±0.02 a	5.0	0.34
7	28	0.19±0.01 cd	9.5	0.46	0.27±0.02 b	13.5	0.48
8	38	0.17±0.01 cd	8.5	1.86	0.36±0.05 c	18.0	1.02
9	52	0.14±0.01 a	7.0	2.13	0.57±0.06 d	28.5	2.61
10	120	0.13±0.02 a	6.5	3.43	0.60±0.03 e	30.0	3.57

Letters compare means of triplicates (composed samples of fifty seeds or 10 pods) in each column by Duncan ($P < 0.05$); – not detected.

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